JOURNAL OF PROTEOMICS 81 (2013) 126–134

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/jprot

Review

Strategies for discovering novel pancreatic cancer biomarkers☆

Alison Chan[a,b], *Eleftherios P. Diamandis*[a,b,c], *Ivan M. Blasutig*[a,c,*]

[a]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada*
[b]*Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada*
[c]*Department of Clinical Biochemistry, University Health Network, Toronto, Ontario, Canada*

ARTICLE INFO

Available online 28 September 2012

Keywords:
Pancreatic cancer
Biomarkers
Screening
Diagnostics

ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in both men and women in Canada and the United States and has the most dismal survival rates among any solid malignancy. Most patients are diagnosed with pancreatic cancer once the disease has progressed into an advanced or metastatic stage, making the only curative approach of resection surgery impossible. The persistent delayed or missed diagnosis of pancreatic cancer can be attributed to the absence of early symptoms and the lack of efficient non-invasive screening or diagnostic tests in clinical practice. Given that earlier diagnosis is critical for ameliorating patients' survival rates, there is an urgent need for biomarkers with enough sensitivity and specificity to help diagnose pancreatic cancer early. Serological biomarkers provide a minimally invasive and efficient way of detecting pancreatic cancer, however, there is currently no marker with sufficient diagnostic sensitivity and specificity to identify early cancer patients. This review focuses on the classical tumor markers for PDAC as well as emerging markers. In addition, we will discuss an integrative proteomic approach used in our lab to identify a panel of biomarkers that have the potential to allow the early detection of PDAC.
This article is part of a Special Issue entitled: From protein structures to clinical applications.

Contents

Abbreviations: PDAC, Pancreatic ductal adenocarcinoma; CT, computerized tomography; EUS, endoscopic ultrasound; ERCP, endoscopic retrograde cholangiopancreatography; CA, Carbohydrate antigen/cancer antigen; CEA, carcinoembryonic antigen; EGTM, European Group on Tumor Markers; ASCO, American Society of Clinical Oncology; NACB, National Academy of Clinical Biochemistry; MUC, mucin; DIGE-MS/MS, difference gel electrophoresis and tandem mass spectrometry; FDA, Food and Drug Administration; APRIL, a proliferation-inducing ligand; TNF, tumor necrosis factor; PanIN, pancreatic intraepithelial neoplasia; HSP, heat shot protein; ULBP2, UL16 binding protein 2; CM, Conditioned media; HPDE, Human pancreatic ductal epithelial cell line; ELISA, Enzyme-linked immunosorbent assays; ROC, receiver operating characteristic; ARG2, anterior gradient homolog 2; OLFM4, olfactomedin-4; SYCN, Syncollin; PIGR, polymeric immunoglobulin receptor; COL6A1, collagen alpha-1 (VI) chain; AUC, area under curve; SCX, strong cation exchange.

☆ This article is part of a Special Issue entitled: From protein structures to clinical applications.

* *Corresponding author at: c/o Dr.* E.P. Diamandis, Toronto General Hospital, University Health Network, 200 Elizabeth St, Room 3EB-362, Toronto, ON, Canada M5G 2C4. Tel.: +1 416 340 4800x7199; fax: +1 416 340 4214.
E-mail addresses: ediamandis@mtsinai.on.ca (E.P. Diamandis), ivan.blasutig@uhn.ca (I.M. Blasutig).

1874-3919/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jprot.2012.09.025

1. Introduction

Despite the increase in research interest and advancement in the understanding of pancreatic cancer over the past few decades, it remains one of the deadliest solid malignancies affecting mankind. It has a 5-year relative survival rate of less than 5%, a median survival rate of less than 6 months and is the fourth leading cause of cancer-related deaths in men and women in Canada and the United States despite being only the tenth most common form of cancer [1,2]. The mortality is almost identical to its incidence rate for this devastating disease and it is estimated that there will be 43,920 new cases and 37,390 deaths from the disease in the U.S. in 2012 [1]. There are several types of pancreatic cancer, the most common being pancreatic ductal adenocarcinoma (PDAC), which accounts for approximately 90% of all pancreatic cancers [3] and for which this review will focus on.

The poor prognosis of PDAC is the result of its silent nature, high metastatic potential and resistance to conventional therapies. To date, the only potentially curative treatment is surgical resection, with the overall five-year survival rate improving to 40% if the tumor is detected at less than 20 mm and to 75% when tumors are detected at less than 10 mm [4]. Unfortunately only approximately 20% of the PDAC patients have their cancers detected at a stage at which surgical resection remains a viable option [5]. Once the disease has progressed into an advanced stage, chemotherapy, radiation or any combinatorial therapies are mostly palliative and have minimal improvement on the patient survival [6].

The inability to detect pancreatic cancer in its early treatable stage is a critical clinical problem. Unfortunately, early PDAC is characterized by a lack of clinical symptoms and when symptoms are present they are generally non-specific (back pain, weight loss, and digestive problems) and do not lead to disease detection. Although a standardized screening strategy is still maturing, the current screening practice commonly includes high risk individuals carrying genetic abnormalities associated with pancreatic cancer, with more than 2 first degree relatives diagnosed with pancreatic cancer, however, such patients only account for less than 5% of all pancreatic cancer [7]. Increasing evidences have shown that new-onset diabetes is present in approximately half of the pancreatic cancer patients, and its occurrence is prevalent even in early stage, asymptomatic pancreatic cancer patients [7]. Therefore, new-onset diabetes may represent a high risk population group to screen for asymptomatic pancreatic cancer [7]. Given that type 2 diabetes is common and pancreatic cancer is rare in the general population, screening all new-onset diabetic patients for pancreatic cancer is not cost-effective without a reliable marker to differentiate between pancreatic cancer-associated diabetes from the more prevalent type 2 diabetes [7]. There have been studies attempting to identify potential candidate biomarkers for pancreatic cancer-associated diabetes, however there is currently no specific marker available since they were either unsuccessful in consequent validation studies or remain to be validated [7–10]. Even if such a marker is found, it may not detect pancreatic cancer in patients without pancreatic cancer-associated diabetes. This leads to the urgent need of the discovery and validation of biomarkers that can help detect PDAC at an early stage in all patients and improve the survival of pancreatic cancer patients.

Although it has been commonly believed that pancreatic cancer progresses and develops metastases too rapidly for early detection to be practical, new research indicates otherwise. Two recent studies analyzing the progression of PDAC using mathematical analyses of tumor genetic sequencing data showed that it may take up to about 10 years after tumor initiation for pancreatic cancer cells to acquire the metastatic capacity to spread to distant organs [11,12]. Based on this finding, there appears to be a long window of opportunity for the detection of pancreatic cancer at an early stage, reinforcing the importance for researchers to discover and validate novel methods for the early detection of pancreatic cancer (Fig. 1).

Diagnostic tests of pancreatic cancer include computerized tomography (CT) scan, endoscopic ultrasound (EUS) and endoscopic retrograde cholangiopancreatography (ERCP) [13,14]. Owing to the fact that these imaging parameters are costly, potentially invasive and time consuming, they are usually performed only after the onset of symptoms. These imaging methods are powerful, yet they are not designed to detect early premalignant lesions, or PDAC when the tumor is small and potentially resectable. In addition, it is often difficult to differentiate chronic pancreatitis from pancreatic cancer. Due to their low cost, and minimal invasiveness, serum based biomarkers remain an ideal method for which to detect PDAC in its early stages. The past decade has seen a plethora of advancements in the field of proteomics, which coupled to the interest in early PDAC detection, and has led to numerous publications on the identification of potential biomarkers for clinical use in pancreatic cancer detection. This review focuses on the most widely used PDAC biomarker CA19-9, emerging novel protein markers and our identification of a biomarker panel using an integrative proteomic approach.

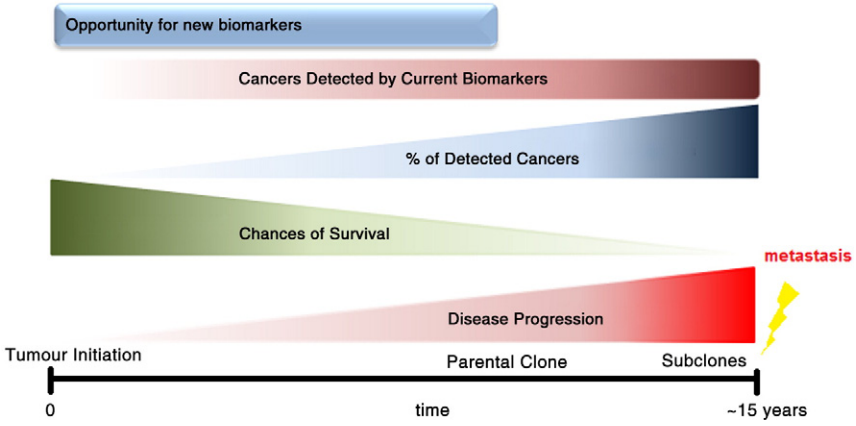

Fig. 1 – The need for additional new biomarkers. Two collaborative investigating groups suggested that it takes up to approximately 15 years from tumor initiation to the development of a parental clone, which eventually develops into subclones with metastatic potential. The chances of survival decline rapidly once the disease has progressed into later stages. The poor prognosis of this devastating disease is partly attributed to the poor diagnostic value of the currently used biomarkers, resulting in the majority of patients being diagnosed at a point at which the tumor has acquired metastatic potential. Therefore, there is an urgent unmet need of new biomarkers to detect pancreatic cancer in its early stages to improve patient survival.

2. Conventional serum markers for pancreatic cancer

Current serum markers used for pancreatic cancer include carbohydrate antigen CA19-9 (Table 1) and carcinoembryonic antigen (CEA). Below we will discuss their discovery, performance characteristics and clinical utility. This will serve to highlight the usefulness of the currently available markers, while demonstrating the clear need for the identification of new markers.

2.1. *Carcinoembryonic antigen CEA*

CEA is a glycoprotein discovered in 1965 by Gold and Freedman in human colon cancer [15]. CEA was the first tumor marker of any clinical value for the detection of pancreatic cancer. An analysis summarizing 13 published reports totaling 1323 patients found that CEA shows a median sensitivity of 54% and a median specificity of 79% [16]. The low sensitivity precludes its ability to be used for the screening of PDAC. In addition, as many tumor types such as breast, stomach, and colorectal also express CEA, it does not have adequate specificity to be used as a detection or monitoring biomarker for pancreatic cancer [17]. As a result, during the last 20 years, CEA has been replaced by CA19-9 which has better diagnostic performance [18].

Given that CEA is not to be used alone, its clinical usefulness may lie in its combination with other biomarkers as a panel for early detection purposes. When used together with CA19-9 in 123 pancreatic cancer patients and 58 patients with benign pancreatic diseases (chronic pancreatitis, pancreatic pseudocyst), their sensitivity and specificity were 86% and 72% respectively, showing an improvement on the sensitivity of either marker alone [19]. However, insufficient data is available to justify its use in the diagnosis of pancreatic cancer even when combined with CA19-9 [20,21].

Table 1 – CA19-9 advantages and disadvantages.

Advantages	Disadvantages
Can be used to monitor patients under therapy	Not suitable for screening or diagnosis
Can be used for assessing prognosis (overall survival)	Lewis blood group-dependent (is not produced in patients with Le^{a-b-} phenotype, regardless of tumor burden)
It provides additional information for therapeutic decisions	Not specific to pancreatic cancer elevated in benign disorders

2.2. *Carbohydrate antigen CA19-9*

The carbohydrate antigen CA19-9 was discovered in 1981 in the search for more reliable and specific diagnostic markers than CEA levels in gastrointestinal cancers [22,23]. CA19-9 was identified as a tumor-associated antigen in a screen of monoclonal antibodies produced from mice immunized with the conditioned media from a human colorectal carcinoma cell line. CA19-9 is a monosialoganglioside, also known to be a sialylated Lewis a antigen [24,25]. Although identified as a marker for colorectal cancer, it was soon realized that it performed better as a pancreatic cancer marker. It was approved by the US Food and Drug Administration (FDA) for monitoring patients with pancreatic cancer and has rapidly become the most widely used biomarker for pancreatic cancer.

CA19-9 is the standard serum tumor marker for PDAC and numerous studies have been published on its performance. In two large literature reviews summarizing all the studies, the authors found CA19-9 to have a similar range of sensitivity and specificity, with an overall mean sensitivity of 80% and a

mean specificity of 86% [16,26]. They further concluded that CA19-9 elevation in non-malignant jaundice results in a significant number of false positives and they recommend combining CA19-9 with other markers to improve its specificity.

Given the low incidence rate of PDAC, it is no surprise that CA19-9 is not recommended by the European Group on Tumor Markers (EGTM) [27], the American Society of Clinical Oncology (ASCO) [28] or the National Academy of Clinical Biochemistry (NACB) [29] to be used in the screening of asymptomatic individuals for pancreatic cancer. In addition to its inability to be used for screening, CA19-9 is very limited in its ability to diagnose pancreatic cancer, especially in early stages of the disease. If CA19-9 is to be used for diagnostic purposes, the NACB panel states that the marker should be used as an aid, with an imaging test being required to confirm the diagnosis [29].

An ideal marker should be able to provide both predictive and prognostic information to help guide clinical decisions [30,31]. CA19-9 has been demonstrated to have both prognostic and predictive values in PDAC, with higher levels of CA19-9 being indicative of poorer survival rates and unresponsiveness to adjuvant therapy. Using a cohort of 260 PDAC patients undergoing resection surgery, it was demonstrated that patients with postoperative CA19-9 levels >90 U/ml did not have long-term benefit from adjuvant chemotherapy compared to those with <90 U/ml [32]. Additionally, having a normal perioperative CA19-9 level is associated with the best prognosis, having a 5-year survival rate of 42% [32]. However CA19-9 is only one of the many parameters that should be included for assessing prognosis and treatment decisions, and is not the most important [29].

Another characteristic of any true cancer biomarker is its ability to correlate with disease burden, and thus provide information about disease activity. As a result, most cancer biomarkers can be used for disease monitoring during or after intervention. The serial measurements of CA19-9 after surgical resection, chemotherapy or during palliative chemotherapy, are recommended for disease monitoring. However due to its lack of sensitivity and specificity, it is not recommended to be used alone, and should be used along with other imaging tests to assess patients' response to therapy [29]. In addition, it can only be used in patients who had elevated levels prior to treatment.

Although CA19-9 is the most widely used biomarker for pancreatic cancer, certain caveats should be kept in mind regarding the usage of CA19-9. Firstly, elevation in a variety of non-malignant diseases and other cancer types affects the specificity of CA19-9. Benign diseases such as biliary obstruction, cholecystitis, chronic and acute pancreatitis, cholangitis, obstructive jaundice, and liver cirrhosis are commonly found to be associated with elevated CA19-9 levels [27]. CA19-9 is not only found to be increased in pancreatic cancer, but also increased in other types of gastrointestinal cancers including gastric, colorectal, esophageal and bile duct cancer [27]. Also, CA19-9 is not produced in approximately 5–10% of the Caucasian population with the Le^{a-b-} phenotype, which prevents them from having detectable CA19-9 levels even in an advanced disease stage [25,27,33,34]. Thirdly, CA19-9 has poor sensitivity in early or small-diameter pancreatic tumors. Lastly, although CA19-9 has been used for more than 30 years, there is a lack of an international standard for CA19-9 resulting in irreproducible quantitative measurements of the marker between assays produced by different manufacturers [17,27,35].

3. The quest for novel pancreatic cancer biomarkers

Over the past two decades, vast effort and millions of dollars of investments have been contributed in hopes of discovering biomarkers that could perform clinically superior than CA19-9. Many papers have been reporting novel single markers that hold promise to revolutionize the diagnosis and management of pancreatic cancer. Most of these newly published biomarkers produce promising results in the initial discovery phase, but are either not adequately validated or reported to have unsuccessful validation and thus fail to proceed past the discovery phase. As a result, no marker has been approved for use by the Food and Drug Administration (FDA) in the past 25 years [36].

The discovery and validation of biomarkers is a time-consuming and challenging process, the difficulties of which are often underestimated. Errors and biases occur at all phases of the discovery and validation studies and include pre-analytical factors (population selection, sample collection, processing and storage), analytical factors (aspects of the assay such as its analytical sensitivity, specificity and robustness) and post-analytical factors (such as data overfitting and interpretation) [36]. These biases and errors complicate the process of biomarker discovery and validation, and failure to identify and correct any one of these errors can lead to the "false discovery" of biomarkers. Barriers that preclude biomarkers to be brought into the clinic have been addressed in detail in recent reviews [30,36,37]. Common biases and errors would be avoided if stringent guidelines and methodologies are followed such as the prospective-specimen-collection, retrospective-blinded-evaluation (PRoBE) design [31] and the Standards for Reporting of Diagnostic Accuracy (STARD) statement [38]. Despite the large number of potential pitfalls present in biomarker discovery studies, there have been thorough and well planned studies performed which have identified seemingly useful biomarkers for pancreatic cancer.

4. Emerging biomarkers

In the past 5 years, over 20 papers have been published reporting on the discovery of novel PDAC biomarkers. Most have not advanced past the initial discovery phase and have very little or no data supporting their clinical usefulness, however, others do have some data supporting their clinical usefulness and for which further validation studies are warranted. Below we will focus on several of these novel markers (Table 2).

DJ-1 was originally identified as a novel oncogene that transformed mouse NIH3T3 cells when cooperating with activated RAS [39]. There is increasing evidence that DJ-1 is involved in AKT activation, which plays an important role in chemoresistance and tumor development. Indeed, proteomic analysis of lung cancer cell lines showed that DJ-1 may be a

Table 2 – Summary of emerging markers of pancreatic cancer.

Marker	Assay used	Sensitivity (%)	Specificity (%)	Sample #	Reference
DJ-1	ELISA	79	79	49 PDAC, 43 CP, 40 healthy	[39,43]
	ELISA	77	87	128 PDAC, 62 healthy	[39]
APRIL	ELISA	70	86	67 PDAC, 55 benign	[44]
Plectin-1	Western blot	87	98	41 PDAC, 15 CP, 4 normal	[47]
PAM4	Immunoassay	82	95	68 PDAC, 19 healthy	[51]
ULBP2	Bead-based immunoassay	84	74	154 PDAC, 142 healthy (bead-based immunoassay)	[53,54]
HSP 70	Immunoelectrophoresis	74	90	23 PDAC, 12 CP, 10 normal	[53]
CA19-9+ICAM-1+OPG CA19-9+CEA+TIMP-1	Bead-based immunoassay	78	94	173 PDAC, 120 healthy	[59]
		71	89	173 PDAC, 70 benign	

ELISA, enzyme-linked immunosorbent assays; PDAC, pancreatic ductal adenocarcinoma; CP, chronic pancreatitis.

chemoresistance-related gene [40]. Another group suggested that DJ-1 correlates with tumor invasion and metastasis in PDAC through the SRC/extracellular signal-regulated kinase/urokinase plasminogen pathway [41]. DJ-1 knock-down in two PDAC cell lines showed reduction in cell migration and invasion ability *in vitro*, and inhibition of metastasis *in vivo* [41]. Immunohistochemistry staining showed up-regulation of DJ-1 in 68.5% of PDAC specimens (n=76), positive correlation with PDAC stages and was able to predict PDAC invasion [41]. Studies have also reported up-regulated levels of DJ-1 in pancreatic juice of pancreatic cancer patients by the difference gel electrophoresis and tandem mass spectrometry (DIGE-MS/MS), and was also found elevated in pancreatic cancer tissue by immunohistochemistry and Western blot [42]. Recently, two studies have found that serum levels of DJ-1 were elevated in pancreatic cancer patients compared with chronic pancreatitis and healthy individuals [39,43]. One study assessed a sample set comprised of 49 PDAC patients, 43 CP patients and 40 healthy subjects; whereas the second study compared between 128 PDAC cases *versus* 62 healthy controls [39,41,43]. For both serum-based studies, case cohorts consist of tumors from early to late stages, allowing for an evaluation of marker expression correlating to tumor stages.

Another potential tumor marker recently reported is a proliferation-inducing ligand (APRIL), which is a newly identified member of the tumor necrosis factor (TNF) superfamily [44]. TNF is a group of cytokines that play a central role in host defense, inflammation and immune homeostasis [45]. Studies have reported that APRIL is overexpressed in several cancer types, especially those derived from the digestive system, and plays a role in mediating tumor progression and invasion both *in vitro* and *in vivo* [46]. To determine if the serum levels of APRIL can be used to detect pancreatic cancer, serum levels of APRIL were examined, together with CEA and CA19-9, in 67 pancreatic cancer patients and 55 patients with benign pancreatic diseases [44]. It was found that APRIL is increased in pancreatic cancer patients and showed a positive correlation with CEA and CA19-9. They showed a sensitivity and specificity of 70.1% and 85.5% for APRIL alone, 83.6% and 80% when combined with both CEA and CA19-9 and 88.1% and 78.2% when used in combination with only CA19-9 [44]. Although providing promising results, the study was performed in a small sample set, and lacked any patients with stage I pancreatic cancer.

PDAC is believed to progress from precursor lesions called pancreatic intraepithelial neoplasia (PanIN), the most advanced stage (PanIN III) being commonly referred to as carcinoma *in situ* [5,13]. Bausch et al. reported that Plectin-1, is not only able to differentiate malignant PDAC from benign chronic pancreatitis disease, it is also elevated in late PanIN III precursor lesions as compared to early PanIN I/II lesions [47]. Their results showed that this marker may be a biomarker for early premalignant cancers and may also be a specific novel imaging biomarker for PDAC [47]. The strength of their work is the apparent specificity of Plectin-1 for PDAC and high-grade PanIN III lesions [48]. The encouraging results reported by Bausche and colleagues is based primarily on immunohistochemistry; studies to assess whether Plectin-1 can be used as a serum-based marker are warranted.

In an immunohistochemistry study, the anti-MUC1 monoclonal antibody PAM4 was found to not stain normal pancreatic ductal epithelium, but was weakly staining in the early PanIN lesions, PanIN-1A and 1B and strongly staining in invasive PDAC specimens. PAM4-reactive antigen expression was suggested to be associated with the early events of the development of invasive PDAC, and may provide an opportunity for early detection and diagnosis of PDAC [49]. Its sensitivity in detection of PDAC was 82%, with a specificity of 95% in serum from 68 PDAC patients and 19 healthy controls [50,51]. Interestingly, PAM4 showed a stage-dependent diagnostic sensitivity, demonstrating sensitivities of 62%, 86%, 91% in patients with stage 1, stage 2 and advanced stages, respectively [51]. Although there is missing information regarding sample storage, blinding or matching, this study described a PAM4 serum assay that may be promising in detecting early-stage PDACs. Another study also demonstrated that combining PAM4 and CA19-9 resulted in an overall improved sensitivity over either marker alone [52].

Dutta and colleagues reported on HSP70 as a novel serological biomarker for detecting early pancreatic cancer as evaluated by a novel immunoelectrophoresis method developed and validated by the authors [53]. HSP70 was found to be significantly elevated in pancreatic cancer patients (n=23) compared to either chronic pancreatitis patients (n=12) or healthy controls (n=10) [53]. Although HSP70 demonstrated an acceptable sensitivity and specificity of 74% and 90%, it failed to differentiate between patients with pancreatic cancer and those with chronic pancreatitis, which is a major limitation of this

marker. Additionally, comparative CA19-9 levels were not analyzed.

A study done by Chang et al. proteomically profiled the secretome of two pancreatic cell lines and identified ULBP2 as a potential biomarker [54]. It was selected for validation because its mRNA level was significantly elevated in pancreatic cancer tissues [54]. Elevated levels of ULBP2 were observed in pancreatic cancer tissues (n=67) compared to non-malignant counterparts using immunohistochemistry. Additionally, serum levels of ULBP2 were elevated in pancreatic cancer patients (n=154) compared to healthy controls (n=142) demonstrating a sensitivity and specificity of 83.8% and 73.9% [54]. The combination of ULBP2 and CA19-9 resulted in an AUC of 0.910, which was greater than either marker alone [54]. Furthermore, ULBP2 (AUC=0.846) was comparable to CA19-9 (AUC=0.839) in differentiating early pancreatic cancer patients from healthy controls.

Besides studies reporting single novel biomarkers, there have been an increasing number of studies that focused on using biomarker panels to detect PDAC [55–58]. Brand et al. reports their analysis of 83 circulating proteins in sera of patients with PDAC (n=333), compared with benign pancreatic conditions (n=144) and healthy controls (n=227) [59]. Prior to analysis, samples were split randomly into training and blind validation sets. The selected markers in the analysis included those that have been previously shown to demonstrate diagnostic ability for pancreatic cancer (CA19-9, MIC-1, osteoprotegerin, TIMP-1, ICAM-1, SAA, HE4), cytokines, hormones, chemokines, apoptotic factors, and apolipoproteins. In the validation set, the panel of CA19-9, ICAM-1 and osteoprotegerin was best able to discriminate PDAC patients from healthy controls with a sensitivity and specificity of 78% and 94%; whereas the best panel to discriminate PDAC from benign controls was CA19-9, CEA and TIMP-1 with a sensitivity and specificity of 71% and 89% [59]. In comparison to other biomarker panel studies, the strengths of this study are inclusion of a large, well annotated sample size, appropriate disease and control groups, standardized sample processing and thorough data analysis.

5. In-house integrative proteomics for the identification of pancreatic cancer biomarkers

Most discovery-phase papers report biomarkers identified from a single biological source. However, PDAC is a heterogeneous cancer, and thus no single biological material or model is able to recapitulate all aspects of the disease [60]. Using a single type of biological material for discovery will both limit the number of true biomarkers discovered as well as hinder the ability to identify and exclude false discoveries. Since every approach has its own advantages and disadvantages, we, and others, hypothesized that using multiple approaches will complement each other and lead to the discovery of more useful biomarkers [60,61].

Our lab has performed an in-depth proteomic analyses using LC-MS/MS on multiple biological materials and integrated our data with those from several publically available databases to identify promising PDAC biomarker candidates (Fig. 2) [62,63].

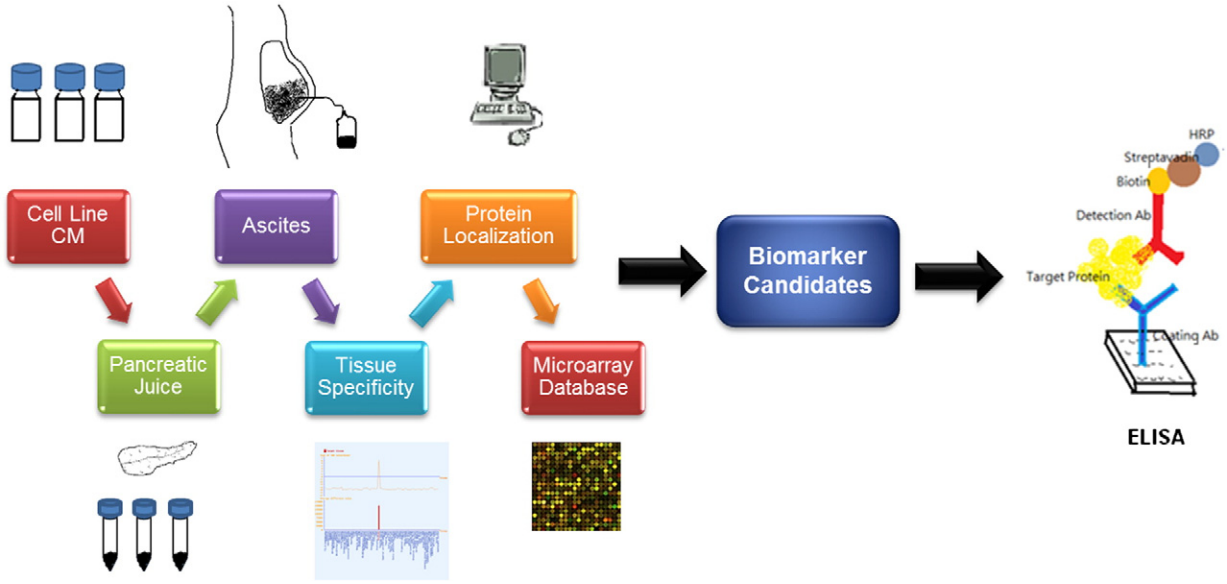

Fig. 2 – Integrative approach in identification of biomarker candidates and follow-up validation study using ELISA. We employed an integrative discovery platform that includes the comparison of proteomes of cell line conditioned media (CM), pancreatic juice, ascites and then filtered our candidates through mining publically available databases, ensuring tissue specificity which enabled the selection of the most promising candidates for verification [62,63]. Of the derived candidates, ELISA, being the gold standard for analyzing serum proteins, was performed in plasma samples from pancreatic cancer patients and healthy controls for preliminary verification [62]. More recently, we identified additional candidates by identifying tissue-specific proteins using bioinformatics [71].

5.1. *Integrative proteomics approach*

Comprehensive proteomic analyses were performed on CM from six pancreatic cancer cell lines, a near-normal human pancreatic ductal epithelial cell line and two pools of pancreatic juice containing a total of six samples from PDAC patients. A total of 3479 non-redundant proteins were identified with high confidence in our combined analysis representing one of the largest and most comprehensive proteomes published on pancreatic cancer-relevant biological fluids in a single study [62].

Selecting promising candidates for verification is a major challenge for most high-throughput discovery studies. In this study, we had developed a multistage approach for prioritizing and generating appropriate candidates utilizing Gene Ontology classifications of protein function, differential protein expression using label-free quantification, comparative analysis with the integration of pancreatic juice and pancreatic cancer ascites fluid proteome and tissue specificity by mining a wide range of publically available databases [62,63].

5.2. *Our in-house protein candidates*

Five candidates, Anterior Gradient Homolog 2 (AGR2), Syncollin (SYCN), Olfactomedin-4 (OLFM4), Collagen alpha-1 (VI) chain (COL6A1) and Polymeric Imunoglobulin Receptor (PIGR) showed a significant increase ($p<0.01$) in the plasma taken from 20 pancreatic cancer patients and 20 healthy controls of similar age and sex [62]. Each of our five protein candidates was able to complement CA19-9 and the combination of our five novel biomarkers also demonstrated the potential to match the sensitivity and specificity of CA19-9 [62].

Given that AGR2 was previously shown to be associated with tumor invasion and metastasis [64,65], its elevation in plasma samples shown by our ELISA data warrants further evaluation of this protein in a larger sample set. In our study OLFM4 was found overexpressed in multiple biological sources, and studies by others have shown that OLFM4 may have a role in proliferation [66]. SYCN is a zymogen granule protein which is shown to be highly specific to the pancreas, however its role in pancreatic cancer remains to be elucidated. Our preliminary verification study has provided evidence that the combination of our five novel biomarkers has the potential to improve on the sensitivity and specificity of CA19-9 for detecting pancreatic cancer. Limitations that we acknowledge in our preliminary verification study are the small sample size and the inclusion of mostly late-stage PDAC patient samples. Hence, we have conducted studies to validate these markers in a larger sample set ($n=432$), which include early stage cancer and benign pancreatic diseases, and some continue to show promise in identifying pancreatic cancer [67].

6. Conclusion and future thoughts

Existing biomarkers for pancreatic cancer are neither sufficiently sensitive nor specific enough to detect early stage disease, or differentiate benign from malignant disease. Although some potential biomarkers show adequate sensitivity, most show poor specificity due to their elevation in other non-cancer specific diseases [60]. Additional serum biomarkers are urgently needed to improve on CA19-9 in pancreatic cancer diagnosis, given that the earlier the patient is diagnosed, the higher the chance of survival. The current status of pancreatic cancer research boasts a number of discovery studies that provide many leads in potential pancreatic cancer biomarkers, but subsequent validation and verification of these leads are lagging. Although many novel biomarkers are discovered, very few turn out to be clinically useful. The problems and difficulties faced in biomarker discovery and validation are abundant and should not be underestimated when designing strategies and experimental studies. Researchers should make sure to use clearly annotated clinical specimens, use appropriate control groups, include large numbers of samples and standardize sample handling in order to generate reliable data [31].

Our discovery efforts utilized an integrated proteomic analysis of multiple biological sources related to pancreatic cancer and identified five proteins which were found to be elevated in plasma from pancreatic cancer patients. Further validation of these five proteins is underway with the aim of developing and validating a biomarker panel for pancreatic cancer.

Although recent estimates on the time course of pancreatic cancer development suggest that the tumor resides for approximately a decade before it metastasizes, evidence from screening studies show that some tumors can progress from a non-invasive to metastatic state within a short period of time [68,69]. One of the primary goals of pancreatic cancer diagnosis is the screening of high risk population to detect pancreatic precursor lesions or early non-invasive pancreatic cancer.

We should also bear in mind that lead time bias can be a major factor affecting the usefulness of potential biomarkers [70]. However, given that the only curative treatment for pancreatic cancer is the surgical resection of early stage disease, there is a great need to identify biomarkers that can detect early pancreatic cancer, or its premalignant lesions, in order to provide the opportunity for this potentially curative surgery to be performed.

REFERENCES

[1] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012;62:10-29.
[2] Hamilton SR, Aalton LA. World Health Organization classification of tumours. Pathology and genetics of tumours of the digestive system; 2000.
[3] Fesinmeyer MD, Austin MA, Li CI, De Roos AJ, Bowen DJ. Differences in survival by histologic type of pancreatic cancer. Cancer Epidemiol Biomarkers Prev 2005;14:1766-73.
[4] Grantzdorffer I, Carl-McGrath S, Ebert MP, Rocken C. Proteomics of pancreatic cancer. Pancreas 2008;36:329-36.
[5] Hidalgo M. Pancreatic cancer. N Engl J Med 2010;362:1605-17.
[6] Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. Nat Rev 2002;2:897-909.
[7] Pannala R, Basu A, Petersen GM, Chari ST. New-onset diabetes: a potential clue to the early diagnosis of pancreatic cancer. Lancet Oncol 2009;10:88-95.
[8] Huang H, Dong X, Kang MX, Xu B, Chen Y, Zhang B, et al. Novel blood biomarkers of pancreatic cancer-associated

diabetes mellitus identified by peripheral blood-based gene expression profiles. Am J Gastroenterol 2010;105:1661-9.
[9] Bartosch-Harlid A, Andersson R. Diabetes mellitus in pancreatic cancer and the need for diagnosis of asymptomatic disease. Pancreatology 2010;10:423-8.
[10] Kolb A, Rieder S, Born D, Giese NA, Giese T, Rudofsky G, et al. Glucagon/insulin ratio as a potential biomarker for pancreatic cancer in patients with new-onset diabetes mellitus. Cancer Biol Ther 2009;8:1527-33.
[11] Campbell PJ, Yachida S, Mudie LJ, Stephens PJ, Pleasance ED, Stebbings LA, et al. The patterns and dynamics of genomic instability in metastatic pancreatic cancer. Nature 2010;467: 1109-13.
[12] Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature 2010;467:1114-7.
[13] Ghaneh P, Costello E, Neoptolemos JP. Biology and management of pancreatic cancer. Gut 2007;56:1134-52.
[14] Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. Lancet 2011;378:607-20.
[15] Gold P, Freedman SO. Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. J Exp Med 1965;121: 439-62.
[16] Goonetilleke KS, Siriwardena AK. Systematic review of carbohydrate antigen (CA 19-9) as a biochemical marker in the diagnosis of pancreatic cancer. Eur J Surg Oncol 2007;33:266-70.
[17] Brody JR, Witkiewicz AK, Yeo CJ. The past, present, and future of biomarkers: a need for molecular beacons for the clinical management of pancreatic cancer. Adv Surg 2011;45:301-21.
[18] Lundin J, Roberts PJ, Kuusela P, Haglund C. The prognostic value of preoperative serum levels of CA 19-9 and CEA in patients with pancreatic cancer. Br J Cancer 1994;69:515-9.
[19] Duraker N, Hot S, Polat Y, Hobek A, Gencler N, Urhan N. CEA, CA 19-9, and CA 125 in the differential diagnosis of benign and malignant pancreatic diseases with or without jaundice. J Surg Oncol 2007;95:142-7.
[20] Del Favero G, Fabris C, Panucci A, Basso D, Plebani M, Baccaglini U, et al. Carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) in pancreatic cancer. Role of age and liver dysfunction. Bull Cancer 1986;73:251-5.
[21] Haglund C, Roberts PJ, Kuusela P, Scheinin TM, Makela O, Jalanko H. Evaluation of CA 19-9 as a serum tumour marker in pancreatic cancer. Br J Cancer 1986;53:197-202.
[22] Koprowski H, Herlyn M, Steplewski Z, Sears HF. Specific antigen in serum of patients with colon carcinoma. Science 1981;212:53-5.
[23] Koprowski H, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P. Colorectal carcinoma antigens detected by hybridoma antibodies. Somatic Cell Genet 1979;5:957-71.
[24] Hanisch FG, Uhlenbruck G, Dienst C. Structure of tumor-associated carbohydrate antigen Ca 19-9 on human seminal-plasma glycoproteins from healthy donors. Eur J Biochem 1984;144:467-73.
[25] Tempero MA, Uchida E, Takasaki H, Burnett DA, Steplewski Z, Pour PM. Relationship of carbohydrate antigen 19-9 and Lewis antigens in pancreatic cancer. Cancer Res 1987;47:5501-3.
[26] Steinberg W. The clinical utility of the CA 19-9 tumor-associated antigen. Am J Gastroenterol 1990;85:350-5.
[27] Duffy MJ, Sturgeon C, Lamerz R, Haglund C, Holubec VL, Klapdor R, et al. Tumor markers in pancreatic cancer: a European Group on Tumor Markers (EGTM) status report. Ann Oncol 2010;21:441-7.
[28] Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, et al. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. J Clin Oncol 2006;24:5313-27.
[29] Goggins M, Koopmann J, Yang D, Canto MI, Hruban RH. National Academy of Clinical Biochemistry (NACB) guidelines for the use of tumor markers in pancreatic ductal adenocarcinoma. [Available at: http://www.aacc.org/sitecollectiondocuments/nacb/lmpg/tumor/chp3i_pancreatic.pdf].
[30] Kulasingam V, Diamandis EP. Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. Nat Clin Pract 2008;5:588-99.
[31] Pepe MS, Feng Z, Janes H, Bossuyt PM, Potter JD. Pivotal evaluation of the accuracy of a biomarker used for classification or prediction: standards for study design. J Natl Cancer Inst 2008;100:1432-8.
[32] Humphris JL, Chang DK, Johns AL, Scarlett CJ, Pajic M, Jones MD, et al. The prognostic and predictive value of serum CA19.9 in pancreatic cancer. Ann Oncol 2012;23(7)1713–2.
[33] Vestergaard EM, Hein HO, Meyer H, Grunnet N, Jorgensen J, Wolf H, et al. Reference values and biological variation for tumor marker CA 19-9 in serum for different Lewis and secretor genotypes and evaluation of secretor and Lewis genotyping in a Caucasian population. Clin Chem 1999;45: 54-61.
[34] Hirano K, Kawa S, Oguchi H, Kobayashi T, Yonekura H, Ogata H, et al. Loss of Lewis antigen expression on erythrocytes in some cancer patients with high serum CA19-9 levels. J Natl Cancer Inst 1987;79:1261-8.
[35] La'ulu SL, Roberts WL. Performance characteristics of five automated CA 19-9 assays. Am J Clin Pathol 2007;127:436-40.
[36] Diamandis EP. Cancer biomarkers: can we turn recent failures into success? J Natl Cancer Inst 2010;102:1462-7.
[37] Pavlou MP, Diamandis EP, Blasutig IM. The long journey of cancer biomarkers from the bench to the clinic. Clin Chem in press. [PMID: 23019307, http://www.ncbi.nlm.nih.gov/pubmed/23019307].
[38] Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al. The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. Clin Chem 2003;49:7–18.
[39] Chen Y, Kang M, Lu W, Guo Q, Zhang B, Xie Q, et al. DJ-1, a novel biomarker and a selected target gene for overcoming chemoresistance in pancreatic cancer. J Cancer Res Clin Oncol 2012;138(9):1463-74.
[40] Zeng HZ, Qu YQ, Zhang WJ, Xiu B, Deng AM, Liang AB. Proteomic analysis identified DJ-1 as a cisplatin resistant marker in non-small cell lung cancer. Int J Mol Sci 2011;12: 3489-99.
[41] He X, Zheng Z, Li J, Ben Q, Liu J, Zhang J, et al. DJ-1 promotes invasion and metastasis of pancreatic cancer cells by activating SRC/ERK/uPA. Carcinogenesis 2012;33:555-62.
[42] Tian M, Cui YZ, Song GH, Zong MJ, Zhou XY, Chen Y, et al. Proteomic analysis identifies MMP-9, DJ-1 and A1BG as overexpressed proteins in pancreatic juice from pancreatic ductal adenocarcinoma patients. BMC Cancer 2008;8:241.
[43] He XY, Liu BY, Yao WY, Zhao XJ, Zheng Z, Li JF, et al. Serum DJ-1 as a diagnostic marker and prognostic factor for pancreatic cancer. J Dig Dis 2011;12:131-7.
[44] Wang F, Chen L, Ding W, Wang G, Wu Y, Wang J, et al. Serum APRIL, a potential tumor marker in pancreatic cancer. Clin Chem Lab Med 2011;49:1715-9.
[45] Wajant H. The role of TNF in cancer. Results Probl Cell Differ 2009;49:1–15.
[46] Wang F, Chen L, Mao ZB, Shao JG, Tan C, Huang WD. Lentivirus-mediated short hairpin RNA targeting the APRIL gene suppresses the growth of pancreatic cancer cells in vitro and in vivo. Oncol Rep 2008;20:135-9.
[47] Bausch D, Thomas S, Mino-Kenudson M, Fernandez-del CC, Bauer TW, Williams M, et al. Plectin-1 as a novel biomarker for pancreatic cancer. Clin Cancer Res 2010;17:302-9.
[48] Collisson E, Tempero M. Blinded by the light: molecular imaging in pancreatic adenocarcinoma. Clin Cancer Res 2010;17:203-5.

[49] Gold DV, Karanjawala Z, Modrak DE, Goldenberg DM, Hruban RH. PAM4-reactive MUC1 is a biomarker for early pancreatic adenocarcinoma. Clin Cancer Res 2007;13:7380-7.
[50] Bunger S, Laubert T, Roblick UJ, Habermann JK. Serum biomarkers for improved diagnostic of pancreatic cancer: a current overview. J Cancer Res Clin Oncol 2011;137:375-89.
[51] Gold DV, Goggins M, Modrak DE, Newsome G, Liu M, Shi C, et al. Detection of early-stage pancreatic adenocarcinoma. Cancer Epidemiol Biomarkers Prev 2010;19:2786-94.
[52] Eguia V, Gonda TA, Saif MW. Early detection of pancreatic cancer. JOP 2012;13:131-4.
[53] Dutta SK, Girotra M, Singla M, Dutta A, Otis Stephen F, Nair PP, et al. Serum HSP70: a novel biomarker for early detection of pancreatic cancer. Pancreas 2012;41:530-4.
[54] Chang YT, Wu CC, Shyr YM, Chen TC, Hwang TL, Yeh TS, et al. Secretome-based identification of ULBP2 as a novel serum marker for pancreatic cancer detection. PLoS One 2011;6:e20029.
[55] Wingren C, Sandstrom A, Segersvard R, Carlsson A, Andersson R, Lohr M, et al. Identification of serum biomarker signatures associated with pancreatic cancer. Cancer Res 2012;72:2481-90.
[56] Chang ST, Zahn JM, Horecka J, Kunz PL, Ford JM, Fisher GA, et al. Identification of a biomarker panel using a multiplex proximity ligation assay improves accuracy of pancreatic cancer diagnosis. J Transl Med 2009;7:105.
[57] Faca VM, Song KS, Wang H, Zhang Q, Krasnoselsky AL, Newcomb LF, et al. A mouse to human search for plasma proteome changes associated with pancreatic tumor development. PLoS Med 2008;5:e123.
[58] Mroczko B, Lukaszewicz-Zajac M, Wereszczynska-Siemiatkowska U, Groblewska M, Gryko M, Kedra B, et al. Clinical significance of the measurements of serum matrix metalloproteinase-9 and its inhibitor (tissue inhibitor of metalloproteinase-1) in patients with pancreatic cancer: metalloproteinase-9 as an independent prognostic factor. Pancreas 2009;38:613-8.
[59] Brand RE, Nolen BM, Zeh HJ, Allen PJ, Eloubeidi MA, Goldberg M, et al. Serum biomarker panels for the detection of pancreatic cancer. Clin Cancer Res 2011;17:805-16.
[60] Kulasingam V, Pavlou MP, Diamandis EP. Integrating high-throughput technologies in the quest for effective biomarkers for ovarian cancer. Nat Rev 2010;10:371-8.
[61] Hanash S, Taguchi A. The grand challenge to decipher the cancer proteome. Nat Rev 2010;10:652-60.
[62] Makawita S, Smith C, Batruch I, Zheng Y, Ruckert F, Grutzmann R, et al. Integrated proteomic profiling of cell line conditioned media and pancreatic juice for the identification of pancreatic cancer biomarkers. Mol Cell Proteomics 2011;10 [M111 008599].
[63] Kosanam H, Makawita S, Judd B, Newman A, Diamandis EP. Mining the malignant ascites proteome for pancreatic cancer biomarkers. Proteomics 2011;11:4551-8.
[64] Barraclough DL, Platt-Higgins A, de Silva Rudland S, Barraclough R, Winstanley J, West CR, et al. The metastasis-associated anterior gradient 2 protein is correlated with poor survival of breast cancer patients. Am J Pathol 2009;175:1848-57.
[65] Ramachandran V, Arumugam T, Wang H, Logsdon CD. Anterior gradient 2 is expressed and secreted during the development of pancreatic cancer and promotes cancer cell survival. Cancer Res 2008;68:7811-8.
[66] Kobayashi D, Koshida S, Moriai R, Tsuji N, Watanabe N. Olfactomedin 4 promotes S-phase transition in proliferation of pancreatic cancer cells. Cancer Sci 2007;98:334-40.
[67] Makawita S, Dimitromanolakis A, Soosaipillai A, Soleas I, Chan A, Gallinger S. Validation of four candidate pancreatic cancer serological biomarkers identifies multiple panels that significantly improve the performance of CA19.9. in preparation.
[68] Goggins M. Markers of pancreatic cancer: working toward early detection. Clin Cancer Res 2011;17:635-7.
[69] Al-Sukhni W, Borgida A, Rothenmund H, Holter S, Semotiuk K, Grant R, et al. Screening for pancreatic cancer in a high-risk cohort: an eight-year experience. J Gastrointest Surg 2011;16: 771-83.
[70] Kay BR, Witte DL. The impact of cancer biology, lead time bias, and length bias in the debate about cancer screening tests. J Insur Med 1991;23:102-4.
[71] Prassas I, Chrystoja CC, Makawita S, Diamandis EP. Bioinformatic identification of proteins with tissue-specific expression for biomarker discovery. BMC Med 2012;10:39.